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13. ABSTRACT (Maximum 200 words) Pseudomonas sp. strain JS42 catalyzes the oxidative removal of nitrite from 2-nitro- toluene to form 3-methylcatechol. This reaction is catalyzed by a multicomponent enzyme system designated 2-nitrotoluene 2,3-dioxygenase. The ferredoxin 2NT component of the dioxygenase system was purified and characterized. It is a Rieske [2Fe-2S] protein with properties similar to the isofunctional ferredoxins in other multicomponent dioxygenase systems. The genes encoding the reductase, ferredoxin and terminal oxygenase components of 2-nitrotoluene dioxygenase were cloned and their nucleotide sequences determined. The predicted amino acid sequences of the three protein components showed significant identity to the sequences of the isofunctional components in naphthalene dioxygenase. However, there were significant differences in the substrate specificities of the two enzymes. The gene encoding catechol 2,3- dioxygenase in a nitrobenzene-degrading Comamonas strain was cloned and expressed in E. Coli. The results to date represent the first purification and characterization of an enzyme system that can oxidatively remove nitro substituents from the benzene nucleus.					
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THIRD ANNUAL TECHNICAL REPORT FOR GRANT

F49620-93-1-0042

Project Officer: Dr. Walter J. Kozumbo

BIODEGRADATION OF NITROAROMATIC COMPOUNDS

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The period covered by this report is 10/31/94-10/31/95

## SUMMARY

*Pseudomonas* sp. strain JS42 catalyzes the oxidative removal of nitrite from 2-nitrotoluene to form 3-methylcatechol. This reaction is catalyzed by a multicomponent enzyme system designated 2-nitrotoluene 2,3-dioxygenase. The ferredoxin<sub>2NT</sub> component of the dioxygenase system was purified and characterized. It is a Rieske [2Fe-2S] protein with properties similar to the isofunctional ferredoxins in other multicomponent dioxygenase systems. The genes encoding the reductase, ferredoxin and terminal oxygenase components of 2-nitrotoluene dioxygenase were cloned and their nucleotide sequences determined. The predicted amino acid sequences of the three protein components showed significant identity to the sequences of the isofunctional components in naphthalene dioxygenase. However, there were significant differences in the substrate specificities of the two enzymes. The gene encoding catechol 2,3-dioxygenase in a nitrobenzene-degrading *Comamonas* strain was cloned and expressed in *E. coli*. The results to date represent the first purification and characterization of an enzyme system that can oxidatively remove nitro substituents from the benzene nucleus.

## RESEARCH OBJECTIVES

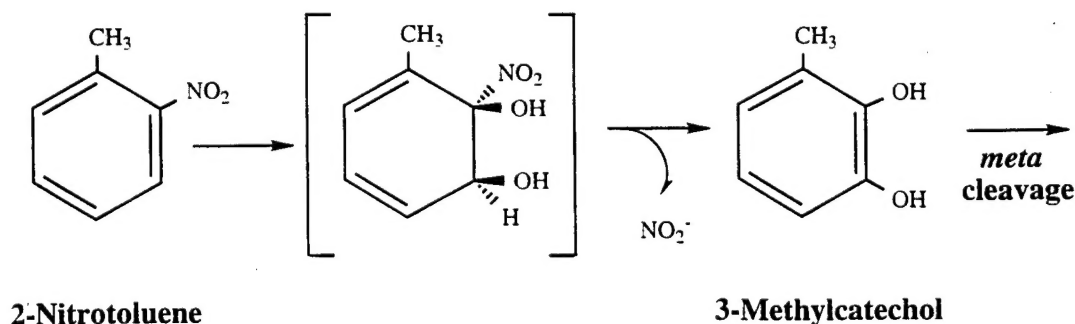
The major objective of this research project is to determine the mechanisms used by bacteria to degrade nitroaromatic compounds. The results obtained will form the scientific foundations necessary for the development of bioremediation technology. The specific aims for the period 10/31/94-10/31/95 were as follows:

1. To purify and characterize component C (ferredoxin<sub>2NT</sub>) of the 2-nitrotoluene dioxygenase complex.
2. To clone, sequence, and express the structural genes of the 2-nitrotoluene dioxygenase complex.
3. To clone and sequence the genes responsible for nitrobenzene oxidation by a *Comamonas* sp. This was another new collaborative venture with Dr. Jim C. Spain's research group at Tyndall Air Force Base, Panama City, FL.

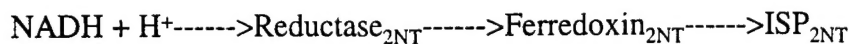
## PROGRESS

*Pseudomonas* sp. strain JS42, isolated by Dr. Jim C. Spain and his colleagues at Tyndall Air Force Base, releases nitrite from 2-nitrotoluene by the reaction shown below. In our second

### Oxidative Pathway for 2NT Degradation in *Pseudomonas* sp. Strain JS42



Annual Technical Report we showed that this reaction is catalyzed by a multicomponent enzyme complex and succeeded in isolating and purifying the protein responsible for the catalytic reaction. This enzyme is an iron-sulfur protein which we have designated ISP<sub>2NT</sub>. We proposed at that time the following organization for the 2-nitrotoluene dioxygenase complex.

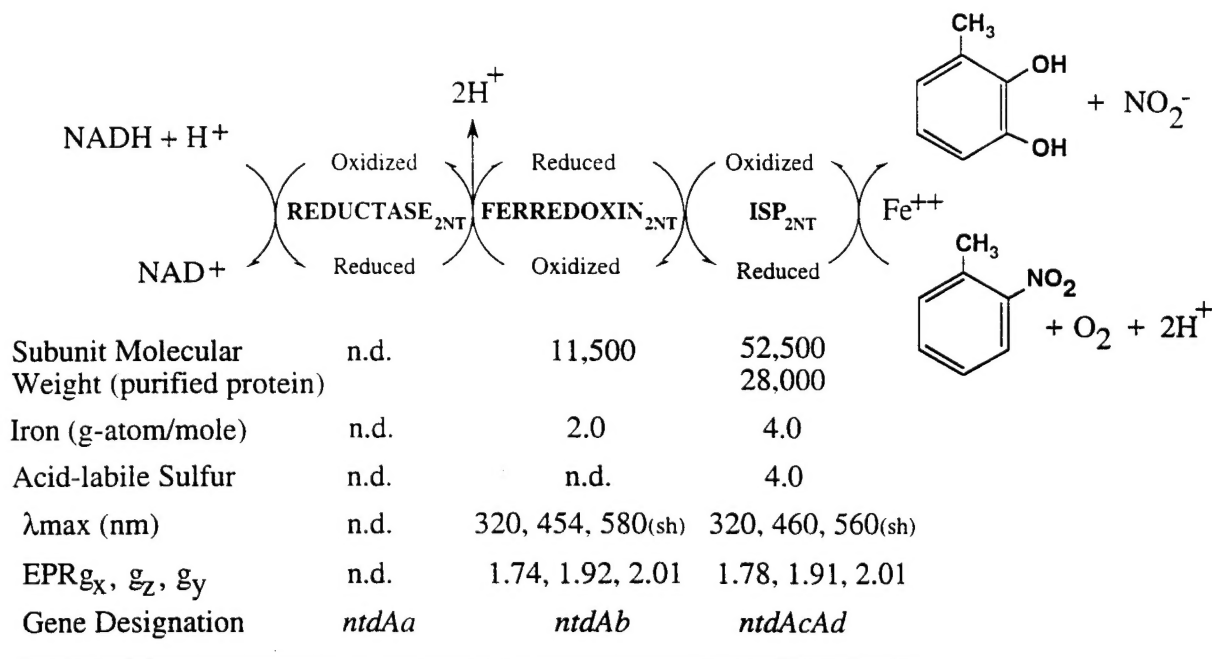


During the current grant period we have almost completed our first and second specific aims and made significant progress on the third. The second protein in the complex, ferredoxin<sub>2NT</sub> has been purified to homogeneity and preliminary characterization studies are complete. A 5-kilobase DNA fragment isolated from strain JS42 was shown to contain all of the genes required for 2-nitrotoluene dioxygenase activity. The nucleotide sequence of the 5-kb fragment was determined and the genes encoding the 2-nitrotoluene dioxygenase components were identified. In spite of considerable effort we have yet to clone the genes responsible for nitrobenzene oxidation by a *Comamonas* sp. A new screening procedure has been developed which will simplify our ability to detect the nitrobenzene oxygenase genes.

Details of progress on the specific aims for the current grant period are given below.

1. **Purification and characterization of the ferredoxin<sub>2NT</sub> a component of the 2-nitrotoluene dioxygenase complex.**

The properties of ferredoxin<sub>2NT</sub> and its relationship to the enzyme complex are shown below.



n.d. = not determined  
sh = shoulder

When we have determined the acid-labile sulfur content of ferredoxin<sub>2NT</sub> this work will be submitted for publication.

## 2. Cloning, expression and nucleotide sequence of the structural genes of the 2-nitrotoluene dioxygenase complex.

A 4.5-kb *SacI* DNA fragment from *Pseudomonas* strain JS42 was cloned in *Escherichia coli* and identified by its ability to release nitrite from 2-nitrotoluene. This showed that all of the structural 2-nitrotoluene dioxygenase genes were located on the 4.9-kb DNA fragment. Both DNA strands were sequenced at the University of Illinois Genetic Engineering Facility (Urbana-Champaign, IL). The nucleotide sequence and the predicted amino acid sequences are shown below.

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GAGCTCGTTGCGCAAGCGCTTCCATCAGTGGGGCATGAAGTACATCTCGCGGATGTCGGTCATTGCCAAGTTGAAGTGCGCGTGCTGGCAAATGGGTGCAAGAGTCACGGGTCGTCA 120
GTGCGCTCTGCAGCGTGTGAGCGCATAGACCACGGGCTCCGCAAGATGCAGTGCATACGGTGTGCGGCTCCATGCCTTTTGTAGGTGCGCAAGAACAATCGTCCTTTAGCGCCGACGCA 240
GCCGTTTAAAGTAATTGCTGACGGCAGGCTCGCTCAGCCCCAGTTTTTCGCGCGCGCTCGATACGCTCCGGTCGAGCAGTAGCTGGTTGAAGACCACCAGCAGATTCAAGTCGATGTCGC 360
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ntdAa M E L V V E P L N L H L N A E T G S T L L D V L R S N E V P I S Y S C M S G
CGCTGCGGCACTTCCGCTTGGCTGTGATTGCGCGGCATCTTCGCGATAACGGCTCCGAGACAGGCGCGCCGAGGCAAGGGGGCTATGCTCGGCTGTGAGCGGTTCTGACC 720
R C G T C R C R V I A G H L R D N G S E T G R P Q A G K G A Y V L A C Q A V L T
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E D C T I E I P E S D E I V V H P A R I V K G T V T A I D E A T H D I R R L R I
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K L A K P L E F S P G Q Y A T V Q F T P E C V R P Y S M A G L P S D A E M E F Q
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I R A V P G G H V S N Y V F N E L S V G A S V R I S G P L G T A Y L R R T H T G
CCCATGCTTTGTGTTGGGGTGAACAGGTCTGGCGCCGCTCCTTTCGATCGTTTCGAGGCGCACTGGAAAGCGGGATGAGCAACCCCATCCATCTGTACTTCCGTTGTGCGGAGCGAGCAG 1200
P M L C V G G G T G L A P V L S I V R G A L E S G M S N P I H L Y F G V R S E Q
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D I Y D E E R L H A L A A R F P N L K V N V V V A T G P A G P G H R S G L V T D
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L I G R D L P N L A G W R A Y L C G A P A M V E A L N L L V A R L G I V P G H I
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H A D A F Y P S G V *
orf2 M S E P Q R L K P V F P Q D
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P K W P G E G S S R V P F W A Y T R E D L Y K R E L E R L F Y A N H W C Y V G L
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E A E I P N P G D F K R T V I G E R S V I M V R D P D G G I N V V E N V C A H C
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E P F E D C A C T T A R *
ntdAb M S E N W I D A I A R D A V
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E N M R V M L K L D *
ntdAc
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T H D S L I P S P G D Y V T A K M G V D E V I V S R Q N D G S V R A F L N V C R
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L Y G D A I K K K C L G L K E V P R I E S F H G F I Y G C F D A E A P P L I D Y

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 L G D A A W Y L E P T F K H S G G L E L V G P P G K V V V K A N W K P F A E N F

TTGTAGGTGACATCTACCACGTTGGTGGACGCACGCAGCGGGTTTGCAGCGAGGGCAGTCGGTATTAGTTCTCTTCCGGGCAACGCTAAGCTTCCACCCGAAGCGCGGGCTTGCAA 3240  
 V G D I Y H V G W T H A A A L R A G Q S V F S S L A G N A K L P P E G A G L Q M

TGACCAGCAAGTATGGCAGTGAATGGGCTTAACGTGGGACTACTACTCCGGTAACCTCAGCGCTGATATGGTTCCTCCGATCTGATGGCATTCCGGCGCCGCAAAACAGGAAAACTCGCCA 3360  
 T S K Y G S G M G L T W D Y Y S G N F S A D M V P D L M A F G A A K Q E K L A K

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 E I G D V R A R I Y R S I L N G T V F P N N S F L T G S A T F K V W N P I D E N

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 V G K S A I G E T S Y R G F Y R A Y Q A H I S S S N W A E F E N A S R N W H T E

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 L T K T T D R \* ntdAd M M I N T Q E D K L V S A H D A E E F H R F F I V Q D

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 D A L L Q E V N T L L T R E A H L L D I Q A Y K A W L E H C V A P E I K Y Q V I

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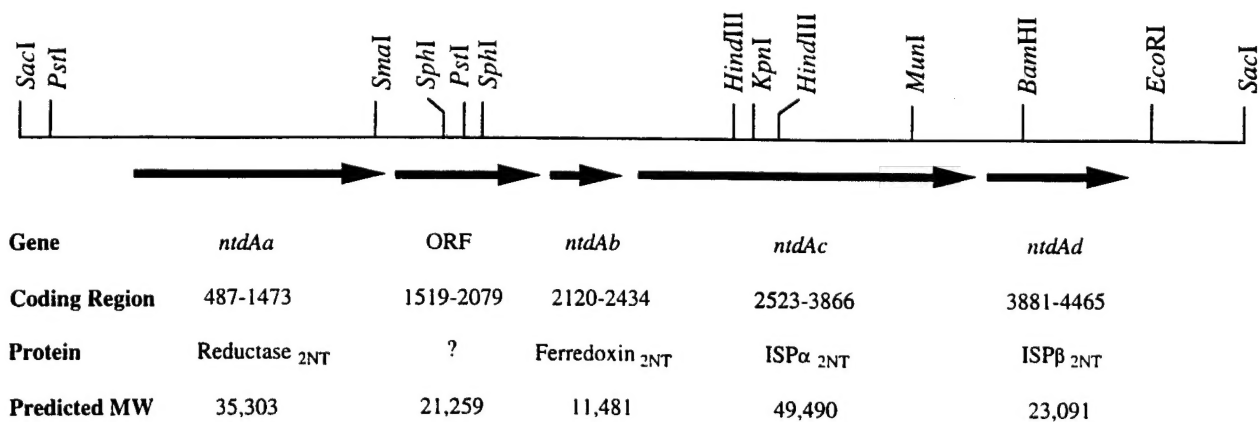
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 H N L L V F L \*

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 CATGCTGAGCATCGATGATGCTTGGGAGAAATTCGCGGCGAGTTTCGACGAGATATTGACATCAACGTCAAAAGCTATTTCAGCGGCATCAGCGCGGCTTCCCGGAGCTC 4912

Nucleotide (nt) sequence of the cloned 4912 bp *SacI* DNA fragment from JS42 containing the genes *ntdAaAbAcAd* required for 2-nitrotoluene dioxygenase activity (top line). The predicted amino acid (aa) sequences for the *ntdAaAbAcAd* genes are also shown (bottom line). Asterisks (\*) indicate stop codons. Potential ribosome binding sites are underlined. The *ntdAaAbAcAd* genes encode the reductase<sub>2NT</sub>, ferredoxin<sub>2NT</sub>, and the ISP<sub>2NT</sub> ( $\alpha$  subunit) and ISP<sub>2NT</sub> ( $\beta$  subunit), respectively. A summary of the results including a restriction map of the 5-kb DNA fragment from JS42 is shown below. This work is currently being prepared for publication.



Gene order and predicted *ntdAaAbAcAd* gene products from *Pseudomonas* sp. JS42

A comparison of the predicted amino acid sequences for reductase<sub>2NT</sub>, ferredoxin<sub>2NT</sub> and ISP<sub>2NT</sub> ( $\alpha$  and  $\beta$  subunits) with other multicomponent dioxygenases is shown below. The 2-nitrotoluene dioxygenase system is clearly related to the naphthalene dioxygenase system from *Pseudomonas* sp. NCIB 9816-4. Since the nucleotide and predicted amino acid sequences for the  $\alpha$  and  $\beta$  subunits of ISP<sub>NAP</sub> from NCIB 9816 have not been published we determined these sequences during the current grant period.

#### 2-Nitrotoluene Dioxygenase (2-NTDO) Amino Acid Sequence Comparisons

	Percent AA Identity <sup>a</sup> with 2-NTDO Predicted Polypeptide			
	Reductase	Ferredoxin	ISP $\alpha$	ISP $\beta$
Naphthalene Dioxygenase (9816-4)	67	72	84	76
Chlorobenzene Dioxygenase (p51)	23	35	34	25
Biphenyl Dioxygenase (RHA1)	18	31	33	26
Biphenyl Dioxygenase (LB400)	19	40	32	30
Biphenyl Dioxygenase (KF707)	19	40	31	27
Toluene Dioxygenase (PpF1)	19	36	34	25

<sup>a</sup> Percent aa identities are the percentage of the amino acids from the 2NTDO component identical to the amino acids of the corresponding three component dioxygenase system. Percentages were determined using the "GAP" program of the Wisconsin Sequence Analysis Package.

We have compared the substrate specificities of naphthalene and 2-nitrotoluene dioxygenases and the results obtained are shown in the table below. The results show that there are significant differences between the two enzymes. It is important to note that 2-nitrotoluene dioxygenase oxidizes naphthalene to almost racemic (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene in contrast to 2,4-dinitrotoluene and naphthalene dioxygenases which form enantiomerically pure preparations of this compound. In addition, 2-nitrotoluene dioxygenase oxidizes the aromatic nucleus of the substrates shown in the following table whereas 2,4-dinitrotoluene and naphthalene dioxygenases oxidize the methyl substituents of most of these substrates.



# Biotransformation Products by 2-Nitrotoluene Dioxygenase and Naphthalene Dioxygenase

Substrate	Products	
	2-Nitrotoluene Dioxygenase	Naphthalene Dioxygenase
2-Nitrotoluene	3-Methylcatechol (18.0) <sup>a</sup> 2-Nitrobenzyl alcohol (1.0)	2-Nitrobenzyl alcohol
3-Nitrotoluene	3-Nitrobenzyl alcohol (4.0) 3-Methylcatechol (1.0)	3-Nitrobenzyl alcohol
4-Nitrotoluene	No products detected	4-Nitrobenzyl alcohol
Nitrobenzene	Catechol	No products detected
Naphthalene	<i>cis</i> -Naphthalenediol (12.6) <sup>b</sup> 1-Naphthenol (1.0)	<i>cis</i> -Naphthalenediol <sup>c</sup>
Indan	1-Indanol (15.0) 3-Hydroxy-1-indanone (5.0) 2-Hydroxy-1-indanone (3.0)	1-Indanol (19.2) 1-Indenol (5.7) <i>cis</i> -Indandiol (2.0) 1-Indanone (1.0)
Indene	<i>cis</i> -Indandiol (9.3) <i>trans</i> -1,2-Dihydroxyindan (1.7) 1-Indenol (1.0) 1-Indanone (trace)	1-Indenol (2.0) <i>cis</i> -Indandiol (1.0)
Trifluorotoluene	Trifluorotoluenediol	No products detected

a) Relative ratios of biotransformation products are in parentheses and were determined by integration of the total ion current chromatograms obtained by GC/MS analysis.

b) 42% enantiomeric excess.

c) 98% enantiomeric excess.



### 3. To clone and sequence the genes responsible for nitrobenzene oxidation by a *Comamonas* sp., strain JS765.

This organism, isolated in Dr. Jim C. Spain's laboratory is novel in terms of the reaction used to initiate the degradation of nitrobenzene. To date it is the only organism known to oxidatively remove nitrite from nitrobenzene. During the current grant period we have tried to use Southern hybridization techniques to identify the nitrobenzene dioxygenase genes in the *Comamonas* strain. To date, no positive hybridization results have been obtained, indicating that the genes responsible for the removal of nitrite from nitrobenzene in this organism are different to any known strains capable of the oxidative degradation of nitroaromatic compounds. It is well known that many dioxygenases will oxidize indole to indoxyl which oxidizes spontaneously to form indigo. Thus the formation of blue colonies in the presence of indole can be used in some cases to indicate the presence of active dioxygenase enzymes. Nitrobenzene grown cells do not oxidize indole to indigo. Consequently, we prepared a cosmid library in pHC79 and sprayed isolated colonies with a solution of catechol in ether. The rationale for this approach is as follows. Catechol is formed from nitrobenzene by the *Comamonas* strain. Ring cleavage of catechol by catechol 2,3-dioxygenase leads to the formation of 2-hydroxymuconic semialdehyde which is bright yellow at alkaline pH due to its high extinction coefficient at 375 nm. Thus recombinant *E. coli* strains which form yellow colonies from catechol will contain JS42 DNA that encodes catechol 2,3-dioxygenase. If the genes for nitrobenzene degradation are located on an operon, or form part of a gene cluster, the isolation of the DNA that contains the catechol 2,3-dioxygenase genes may also contain the genes encoding nitrobenzene dioxygenase. Our initial experiments resulted in the cloning of a large fragment (40-45-kb) of JS42 DNA into the cosmid vector pHC79. This recombinant plasmid, designated pDTG900, contains the gene for catechol 2,3-dioxygenase. Subcloning experiments with the vector pK19 gave a recombinant strain of *E. coli* DH5 $\alpha$  (pDTG901) which turned yellow when sprayed with an ether solution of catechol. The size of the insert in pDTG901 was approximately 7-kb.

### 4. Significance.

The results obtained to date represent the first demonstration of the multicomponent nature of an enzyme responsible for initiating the degradation of a nitroaromatic pollutant. The cloning of the genes encoding the structural components of 2-nitrotoluene dioxygenase is a major breakthrough which will enable us to clone each component in high expression vectors. This is particularly important for genes *ntdAc* and *ntdAd* which encode the large ( $\alpha$ ) and small ( $\beta$ ) subunits of the oxygenase component. Thus, we will be able to determine the function of each subunit, locate the active site of the enzyme and ultimately elucidate the mechanism of nitrite release. These are high but not unobtainable goals. The rapid advances in bioanalytical technology including X-ray crystallography, electrospray and laser desorption techniques in mass spectrometry, NMR, EPR, resonance Raman, Mössbauer, ENDOR and EXAFS spectroscopies can all be utilized to study the mechanism of action of the enzyme. Knowledge from these experiments can be used to construct a more efficient enzyme with the ability to degrade a wide range of nitroaromatic pollutants. This type of information is a necessary adjunct to ongoing developments in bioremediation technology. It lends credibility and direction to applied studies and could conceivably lead to the development of stable synthetic catalysts that can be used to remove nitroaromatic pollutants from contaminated sites. These are the ultimate goals of this project and they focus on the challenges posed by the presence of nitroaromatic compounds in military establishments and the environment.

## PARTICIPATING PROFESSIONALS

David T. Gibson, Ph.D.

John M. Brand, Ph.D.

Juanito V. Parales, B.S.

Ashwani Kumar, Ph.D.--participated in project but did not receive financial support.

Rebecca E. Parales, Ph.D.--participated in project but did not receive financial support.

## PUBLICATIONS

None (3 in preparation).

## INTERACTIONS

### a. Participation/presentations.

i. David T. Gibson. "**Multiple reactions catalyzed by multicomponent dioxygenases.**" Presented at a Keystone Symposium on Environmental Biotechnology, March 16-22, 1995. Lake Tahoe, Granlibakken Resort, California.

ii. David T. Gibson. "**New insights on the bioconversion of contaminants by oxygenases.**" Gordon Research Conference on Applied and Environmental Microbiology, July 2-7, 1995. New Hampton School, New Hampton, New Hampshire.

iii. David T. Gibson has been invited to present a seminar on "**Microbial Solutions to Problems of Environmental Pollution**" to the Environmental Division of DuPont Central Research and Development, Wilmington, DE, on May 1, 1996. Contact: Dr. Vasantha Nagarajan.

iv. Rebecca E. Parales presented a poster, "**2-Nitrotoluene Dioxygenase from *Pseudomonas* sp. Strain JS42: Protein Purification, Molecular Biology, and Substrate Specificity**", at the Fifth Annual Symposium on *Pseudomonas* held August 21-26, 1995, at Tsukuba, Japan. Co-authors contributing to this work were Dr. Danmei An, Dr. Ashwani Kumar, Mr. Juan V. Parales, Mr. Sol M. Resnick, Dr. Jim C. Spain, and Dr. David T. Gibson.

### b. Consultative and advisory functions.

i. **Industry: Genencor International.** Properties and crystallization of hydrocarbon dioxygenase components. Primary contact: Dr. Gregory M. Whited (visit 5/15/95 and several telephone and e-mail contacts).

ii. **Government: Los Alamos National Laboratory.** Invitation to participate with 5-8 other experts in a discussion with scientists at the Los Alamos National Laboratory September 25 and 26. The purpose is to identify research problems in bioremediation which are best suited to a multidisciplinary group of scientists at Los Alamos with expertise in structural biology, theory and modeling, molecular biology, and microbial ecology. Unable to attend due to health problems.

iii. **Air Force: Tyndall Air Force Base, Panama City, FL.** We have had an ongoing consultation/collaboration relationship with Dr. Jim C. Spain's laboratory for several years. This year it became clear that both laboratories had complementary expertise in a variety of ongoing research projects. In order to optimize the transfer of information and to identify areas of mutual interest that could serve as the starting point for new collaborative projects, a one day workshop on the Biodegradation of Nitroaromatic Compounds was held on 9 June, 1995. The program for the workshop is given below.

## BIODEGRADATION OF AROMATIC COMPOUNDS

### Reduction of nitro groups

- 9:00 Chuck Somerville- Preparing nitrobenzene for ring cleavage: the reductive pathway
- 9:15 John Davis- Genetic analysis of the nitrobenzene reduction pathway
- 9:30 Urs Lendenmann- Purification and characterization of 2-aminophenol dioxygenase
- 9:45 Andreas Schenzle- 3-Nitrophenol degradation by *Alcaligenes eutrophus* JMP134
- 10:00 Paul Fiorella- Synthesis of novel TNT metabolites by strain JS45

### Dioxygenase catalyzed removal of nitro groups

- 10:30 Wen Chen Suen, Billy Haigler- 2,4-Dinitrotoluene degradation: pathway, gene cloning and sequence analysis
- 10:50 Shirley Nishino- Recalcitrance of 2,6-DNT
- 11:05 Juan Parales, Wen Chen Suen- 2-Nitrotoluene dioxygenase sequence: comparison with DNT dioxygenase and naphthalene dioxygenase
- 11:20 Juan Parales, Sol Resnick- 2-Nitrotoluene dioxygenase: Substrate specificity
- 11:35 Becky Parales-  $ISP_{NAP}$ : Random mutagenesis of the ISP small subunit gene
- 11:50 Haiyan Jiang-  $ISP_{TOL}$ : Reconstitution of active enzyme from purified  $\alpha$  and  $\beta$  subunits
- 1:30 Billy Haigler, Sol Resnick- 2,4,5-Trihydroxytoluene oxygenase: ortho or meta ring cleavage?
- 1:45 Staci Eaton- Dihydronaphthalene metabolism by *Beijerinckia*
- 2:00 Lloyd Nadeau- Transformation of DDT by *Alcaligenes eutrophus*. 3-nitrobenzoate dioxygenase

### Oxidative degradation of nitrobenzene

- 2:15 Shirley Nishino- Pathway and substrate range
- 2:30 Becky Parales- Cloning of genes and selection of mutants: thoughts and future plans
- 2:40 Kadiyala Venkateswarlu- Biodegradation of PNP by *Bacillus sphaericus*
- 3:15 Small group discussions

## INVENTIONS/PATENT DISCLOSURES

None.

## **FUTURE STUDIES**

Specific Aims for the next grant period:

1. Determination of the stoichiometry of the reaction catalyzed by 2-nitrotoluene dioxygenase.
2. Initiate studies on the function of the open reading frame in the nucleotide sequence encoding the structural genes of 2-nitrotoluene dioxygenase.
3. Construction of hybrid dioxygenases from  $ISP_{2NT}$  and  $ISP_{NAP}$  to determine regions responsible for substrate specificity.